The Interconnected Web: Functional, Structural, and Regulatory Linkages Among Human mtDNA-Encoded Proteins

I. Introduction: The Human Mitochondrial Genome and its Protein-Coding Repertoire

A. Overview of Human Mitochondrial DNA (mtDNA)

Human mitochondrial DNA (mtDNA) is a compact, circular, double-stranded molecule, typically approximately 16.6 kilobases (kb) in size, which resides within the mitochondrial matrix. It is almost exclusively maternally inherited and, despite its small size, plays an indispensable role in cellular life. The human mtDNA harbors 37 genes: 13 genes encoding proteins, 22 genes encoding transfer RNAs (tRNAs), and 2 genes encoding ribosomal RNAs (rRNAs). These RNA components are essential for the mitochondrial translational machinery, which synthesizes the 13 mtDNA-encoded proteins. The genome is organized into a guanine-rich heavy (H) strand and a cytosine-rich light (L) strand. The H-strand serves as a template for the majority of the coding sequences, including 12 of the 13 protein-coding genes and 14 tRNA genes, while the L-strand encodes one protein (MT-ND6) and 8 tRNA genes. A key characteristic of mtDNA is its critical contribution to cellular energy production; all 13 of its protein products are core, often highly hydrophobic, subunits of the enzyme complexes that constitute the oxidative phosphorylation (OXPHOS) system located in the inner mitochondrial membrane (IMM). The evolutionary retention of this small set of protein-coding genes within the mitochondria, while thousands of others were transferred to the nuclear genome during eukaryotic evolution, underscores their fundamental and indispensable nature for core OXPHOS functions. This limited

repertoire, essential for aerobic energy production, inherently connects these proteins through their shared, critical role. Furthermore, the remarkable compactness of the mtDNA, which largely lacks introns and features minimal intergenic spacers or even overlapping genes, means that the expression and processing of these 13 protein-coding genes are intimately linked from the initial stage of polycistronic transcription, forming a primary layer of connection.

B. The Canonical 13 Protein-Coding Genes and Their Products

The 13 proteins encoded by human mtDNA are all integral components of the OXPHOS system. Their distribution among the OXPHOS complexes is as follows:

- **Complex I (NADH:ubiquinone oxidoreductase):** This complex includes seven mtDNA-encoded subunits:
  - MT-ND1 (NADH-ubiquinone oxidoreductase chain 1)
  - MT-ND2 (NADH-ubiquinone oxidoreductase chain 2)
  - MT-ND3 (NADH-ubiquinone oxidoreductase chain 3)
  - MT-ND4 (NADH-ubiquinone oxidoreductase chain 4)
  - MT-ND4L (NADH-ubiquinone oxidoreductase chain 4L)
  - MT-ND5 (NADH-ubiquinone oxidoreductase chain 5)
  - MT-ND6 (NADH-ubiquinone oxidoreductase chain 6)
- **Complex III (Ubiquinol-cytochrome c reductase or Cytochrome bc1 complex):** This complex contains one mtDNA-encoded subunit:
  - MT-CYB (Cytochrome b)
- **Complex IV (Cytochrome c oxidase):** This complex has three mtDNA-encoded subunits:
  - MT-CO1 (Cytochrome c oxidase subunit I)
  - MT-CO2 (Cytochrome c oxidase subunit II)

    - MT-CO3 (Cytochrome c oxidase subunit III)
- **Complex V (ATP synthase):** This complex includes two mtDNA-encoded subunits:
    - MT-ATP6 (ATP synthase F0 subunit 6)
    - MT-ATP8 (ATP synthase F0 subunit 8)

These 13 proteins are predominantly highly hydrophobic and are integral to the structure and function of their respective enzyme complexes, playing critical roles in electron transport and/or proton translocation across the IMM.

C. Defining "Connection" in the Context of mtDNA-Encoded Proteins

The "connection" between these 13 mtDNA-encoded proteins is not singular but multifaceted, reflecting their deep integration into mitochondrial biology. This report will explore these connections through several lenses:

- **Functional Connection:** Their collective and sequential participation in the biochemical reactions of the OXPHOS pathway, which is essential for cellular ATP production.
- **Structural Connection:** Their co-assembly into discrete multi-subunit enzyme complexes (Complexes I, III, IV, and V) and the further organization of these complexes into higher-order structures known as respiratory supercomplexes.
- **Regulatory Connection:** The coordinated expression of their genes, which involves polycistronic transcription from a limited number of promoters, shared RNA processing machinery, and regulation by common or interacting *trans*-acting factors, many of which are encoded by the nuclear genome.
- **Network Connection:** Broader protein-protein interactions that

these subunits or their respective complexes engage in, linking them to other mitochondrial processes (e.g., quality control, dynamics) and even inter-organellar communication pathways.
- **Pathophysiological Connection:** The observation that defects in one mtDNA-encoded protein, its assembly, or its regulation often have cascading effects on other components of the OXPHOS system, leading to mitochondrial dysfunction and a spectrum of human diseases.

Understanding these diverse connections is crucial for a comprehensive appreciation of mitochondrial function in health and disease.

II. Beyond the Canonical Thirteen: Expanding the Mitochondrial Proteome?

A. Evidence for Additional mtDNA-Encoded Functional Peptides

While the focus of mitochondrial research has historically been on the 13 canonical protein-coding genes, emerging evidence suggests that the human mitochondrial genome may possess a larger functional repertoire than previously believed. Studies have identified additional open reading frames (ORFs) within mtDNA that could encode functional peptides, potentially expanding the list of mitochondrially derived protein products.

Notably, **Humanin (HN)** is a 24-amino acid peptide whose coding sequence has been identified within the gene for the 16S ribosomal RNA (MT-RNR2). Humanin has garnered significant attention due to its reported cytoprotective functions. At an intracellular level, HN is implicated in mitochondrial-nuclear retrograde signaling and interacts with pro-apoptotic proteins to prevent apoptosis. Extracellularly, HN appears to regulate critical cellular processes

including survival, metabolism, and inflammation. The discovery of Humanin originating from an rRNA gene highlights a potential for cryptic information within the mitochondrial genome.

Another candidate is **GAU (Gene Antisense Ubiquitous)**, a putative protein-coding gene located on the complementary (light) strand, antisense to the *MT-CO1* gene. Analysis suggests that the *GAU* ORF is evolving under purifying selection and that the deduced GAU proteins share conserved amino acid signatures, supporting its potential functionality as a protein-coding gene.

Beyond Humanin and GAU, bioinformatics analyses have suggested the presence of other ORFs with potential functionality in the 16S rRNA gene, the 12S rRNA gene, and even within the D-loop (the main non-coding region of mtDNA). The mechanisms that might enable the expression of these additional peptides could include antisense transcription or alternative translation initiation and frameshifting events, allowing for an expanded coding capacity without a significant increase in genome size.

The existence of such peptides, encoded within regions traditionally considered non-coding or dedicated to RNA production, points towards a higher level of information density and regulatory sophistication within the mtDNA than previously appreciated. This implies that the functional landscape of mitochondrially encoded factors might be broader, potentially involving these novel peptides in modulating the environment or function of the canonical 13 OXPHOS proteins.

B. Implications for Understanding Mitochondrial Genetic Output

The confirmation and full functional characterization of these additional mtDNA-encoded peptides would significantly alter our

understanding of mitochondrial genetic output. These peptides could represent new classes of mitochondrially derived signaling molecules or effector proteins, forging novel connections between the mitochondrial genome and broader cellular physiology. For instance, peptides like Humanin, if indeed involved in retrograde signaling as suggested, would constitute a direct communication pathway from the mitochondrion (reflecting its genetic and functional state) to the nucleus. Such a pathway could influence the expression of nuclear-encoded OXPHOS subunits or the myriad assembly factors and regulatory proteins essential for the biogenesis and function of the complexes containing the 13 canonical mtDNA-encoded proteins. This establishes another layer of connection, mediated by these novel peptides, fostering the intricate dialogue between the mitochondrial and nuclear genomes required for cellular homeostasis. Consequently, the concept of "connection between the 13 proteins" might need to be expanded to encompass the interactions and regulatory networks involving all mtDNA-encoded protein and peptide products.

III. Core Function: The 13 Proteins as Pillars of Oxidative Phosphorylation (OXPHOS)

A. Overview of OXPHOS

Oxidative phosphorylation is the metabolic pathway by which eukaryotic cells generate the vast majority of their adenosine triphosphate (ATP), the universal energy currency. This process takes place on the inner mitochondrial membrane (IMM) and involves a series of five multi-subunit protein complexes (Complexes I-V) and two mobile electron carriers, ubiquinone (Coenzyme Q) and cytochrome c. Electrons derived from the oxidation of nutrients (primarily via NADH and succinate/$FADH_2$) are passed sequentially

through Complexes I/II, III, and IV. This electron transport is coupled to the pumping of protons (H+) from the mitochondrial matrix to the intermembrane space (IMS) by Complexes I, III, and IV, establishing an electrochemical proton gradient (proton-motive force) across the IMM. Complex V (ATP synthase) then utilizes the energy stored in this gradient to drive the synthesis of ATP from ADP and inorganic phosphate (Pi) as protons flow back into the matrix. The 13 canonical proteins encoded by mtDNA are all indispensable core subunits of Complexes I, III, IV, and V, highlighting their central, interconnected role in this vital pathway. Their shared characteristic of being highly hydrophobic integral membrane proteins dictates their localization and critical functions within the membrane-embedded segments of these complexes, often directly participating in transmembrane electron transfer or proton translocation.

B. Complex I (NADH:Ubiquinone Oxidoreductase): The Electron Entry Point

Complex I is the largest and most intricate of the OXPHOS complexes, serving as the primary entry point for electrons from NADH into the electron transport chain.

- **mtDNA-Encoded Subunits:** Seven subunits of Complex I are encoded by mtDNA: MT-ND1, MT-ND2, MT-ND3, MT-ND4, MT-ND4L, MT-ND5, and MT-ND6. These are all highly hydrophobic, transmembrane proteins that form the core of the membrane-embedded arm of Complex I. They are crucial for the ubiquinone reductase activity and, critically, for the translocation of protons across the IMM.
- **Function:** Complex I catalyzes the transfer of two electrons from NADH to ubiquinone (CoQ), reducing it to ubiquinol (CoQH2). This redox reaction is coupled to the pumping of

typically four protons from the mitochondrial matrix to the IMS, contributing significantly to the proton-motive force. The mtDNA-encoded subunits are essential for both the catalytic activity and the structural integrity and assembly of the entire complex.

- **Individual Protein Contributions:**
  - **MT-ND1:** A core subunit essential for the catalytic activity and assembly of Complex I. It is a key component of the proton-pumping machinery.
  - **MT-ND2:** Another core subunit critical for both catalytic activity and the assembly of Complex I.
  - **MT-ND3:** A core subunit essential for the catalytic activity of Complex I. Its expression is notably regulated at the mRNA level by the nuclear-encoded protein FASTKD1.
  - **MT-ND4:** A core subunit vital for the catalytic function and assembly of Complex I.
  - **MT-ND4L:** A smaller core subunit, integral to the membrane arm, and directly involved in the proton translocation pathway.
  - **MT-ND5:** A core subunit indispensable for the catalytic activity and assembly of Complex I.
  - **MT-ND6:** A core subunit essential for catalytic activity and assembly. It plays a particularly crucial role in the formation and stability of the membrane arm of Complex I.
- **Connections within Complex I:** The seven MT-ND proteins are profoundly interconnected, forming the structural and functional backbone of the Complex I membrane arm. Their coordinated expression, insertion into the IMM, and assembly are paramount for the complex's ability to transfer electrons to ubiquinone and pump protons. Dysfunction or absence of any one of these

subunits can severely impair the assembly and function of the entire membrane domain, and consequently, the whole of Complex I, highlighting their stoichiometric interdependence.

C. Complex III (Ubiquinol-Cytochrome c Reductase): The Q-Cycle Hub

Complex III, also known as the cytochrome bc1 complex, occupies a central position in the electron transport chain, mediating electron transfer from ubiquinol to cytochrome c.

- **mtDNA-Encoded Subunit:** Only one subunit of Complex III is encoded by mtDNA: MT-CYB (Cytochrome b). MT-CYB is a transmembrane protein that forms the catalytic core of the complex, containing two distinct heme b groups: heme bL (low potential) and heme bH (high potential).
- **Function:** Complex III catalyzes the oxidation of ubiquinol (produced by Complexes I and II) and the reduction of cytochrome c. This process is intricately coupled to proton translocation across the IMM via a mechanism known as the Q-cycle. For every two electrons transferred to cytochrome c, four protons are typically translocated to the IMS. MT-CYB is absolutely essential for this electron transfer and proton pumping activity.
- **Connections within Complex III:** Although MT-CYB is the sole mtDNA-encoded component, it is the catalytic heart of Complex III. It is structurally and functionally integrated with nuclear-encoded subunits, most notably cytochrome c1 and the Rieske iron-sulfur protein, to execute the complex redox reactions of the Q-cycle. Its central role makes the coordinated expression and assembly of MT-CYB with its nuclear-encoded partners critical for Complex III function.

D. Complex IV (Cytochrome c Oxidase): The Terminal Oxidase

Complex IV is the terminal oxidase of the mitochondrial respiratory chain, responsible for the final transfer of electrons to molecular oxygen.

- **mtDNA-Encoded Subunits:** Three core subunits of Complex IV are encoded by mtDNA: MT-CO1, MT-CO2, and MT-CO3. These three proteins form the catalytic core of the enzyme and are essential for its function.
- **Function:** Complex IV catalyzes the four-electron reduction of molecular oxygen (O2) to two molecules of water (H2O), using electrons donated by reduced cytochrome c. This highly exergonic reaction is coupled to the pumping of protons from the mitochondrial matrix to the IMS, further contributing to the proton-motive force.
- **Individual Protein Contributions:**
  - **MT-CO1:** This is the largest and most central subunit of Complex IV. It houses the catalytic active site, which includes heme a, heme a3, and a copper ion (CuB). The reduction of oxygen to water occurs at this binuclear center (heme a3-CuB).
  - **MT-CO2:** This subunit contains the dinuclear copper center (CuA), which serves as the entry point for electrons from reduced cytochrome c. Electrons are then transferred from CuA to heme a in MT-CO1. The maturation and assembly of MT-CO2 are complex processes requiring dedicated nuclear-encoded assembly factors such as COX20 and COX18.
  - **MT-CO3:** An essential structural component of the catalytic core, MT-CO3 is crucial for the assembly, stability, and

optimal function of Complex IV. There is some evidence suggesting potential interactions of MT-CO3 with proteins outside of Complex IV, such as SNCA (α-synuclein), KRAS, RAC1, and HSPB2, though the functional significance of these interactions in the context of OXPHOS requires further investigation.

- **Connections within Complex IV:** The three mtDNA-encoded subunits of Complex IV are intimately connected, forming a tightly integrated catalytic engine. MT-CO2 acts as the electron acceptor from cytochrome c, MT-CO1 performs the critical oxygen reduction and proton translocation, and MT-CO3 provides essential structural support and contributes to the overall stability and assembly of the enzyme. Their coordinated synthesis and precise assembly with numerous nuclear-encoded subunits are paramount for functional cytochrome c oxidase.

E. Complex V (ATP Synthase): The ATP Factory

Complex V, or F1F0 ATP synthase, is the enzyme responsible for the synthesis of ATP, utilizing the energy stored in the proton gradient established by the respiratory chain complexes.

- **mtDNA-Encoded Subunits:** Two subunits of the F0 sector of ATP synthase are encoded by mtDNA: MT-ATP6 and MT-ATP8. These proteins are integral components of the membrane-embedded F0 domain, which functions as a proton channel.
- **Function:** The F0 domain facilitates the passage of protons from the IMS back into the mitochondrial matrix, down their electrochemical gradient. This proton flow drives the rotation of parts of the F0 and the central stalk, which in turn induces conformational changes in the catalytic F1 headpiece

(composed entirely of nuclear-encoded subunits), leading to the synthesis of ATP from ADP and Pi.
    - **MT-ATP6 (subunit a):** This protein is a major component of the proton channel itself, forming a crucial interface with the ring of nuclear-encoded c-subunits. It is directly involved in the process of proton translocation.
    - **MT-ATP8 (subunit 8 or A6L):** This small, hydrophobic protein is also part of the F0 domain. It is thought to be important for the structural integrity of the F0 motor, potentially linking MT-ATP6 to other F0 subunits or the peripheral stalk, and may play a role in the dimerization and stability of Complex V monomers.
- **Connections within Complex V:** MT-ATP6 and MT-ATP8 are physically and functionally linked within the F0 domain. MT-ATP6 is central to proton conduction, while MT-ATP8 contributes to the overall architecture and stability of the F0 proton channel and its integration with the rest of the ATP synthase complex. Their correct assembly with each other and with multiple nuclear-encoded F0 and F1 subunits is essential for coupling proton flow to ATP synthesis.

The intricate cooperation of these 13 mtDNA-encoded proteins within their respective complexes, and the sequential operation of these complexes, forms the functional backbone of cellular energy production. Electrons flow from Complex I (MT-ND subunits) or Complex II (entirely nuclear-encoded) via ubiquinone to Complex III (MT-CYB), then via cytochrome c to Complex IV (MT-CO subunits). The proton gradient generated by Complexes I, III, and IV is then harnessed by Complex V (MT-ATP6 and MT-ATP8) to synthesize ATP. This sequential functional linkage establishes a profound connection among these distinct groups of mtDNA-encoded proteins, all

contributing to the singular goal of efficient energy conversion.

F. Table 1: The Human mtDNA-Encoded Proteome and OXPHOS Association

mtDNA Gene Name	Protein Name	UniProt ID (Human)	OXPHOS Complex	Brief Core Function
MT-ND1	NADH-ubiquinone oxidoreductase chain 1	P03886	Complex I	Core subunit; essential for catalytic activity, assembly, and proton pumping
MT-ND2	NADH-ubiquinone oxidoreductase chain 2	P03891	Complex I	Core subunit; essential for catalytic activity and assembly
MT-ND3	NADH-ubiquinone oxidoreductase chain 3	P03893	Complex I	Core subunit; essential for catalytic activity
MT-ND4	NADH-ubiquinone oxidoreductase chain 4	P03900	Complex I	Core subunit; essential for catalytic activity,

				assembly, and proton pumping
MT-ND4L	NADH-ubiquinone oxidoreductase chain 4L	P03901	Complex I	Core subunit; part of membrane arm involved in proton translocation
MT-ND5	NADH-ubiquinone oxidoreductase chain 5	P03915	Complex I	Core subunit; essential for catalytic activity, assembly, and proton pumping
MT-ND6	NADH-ubiquinone oxidoreductase chain 6	P03923	Complex I	Core subunit; essential for catalytic activity, assembly of membrane arm
MT-CYB	Cytochrome b	P00156	Complex III	Catalytic core; binds hemes, essential for

				Q-cycle electron transfer and proton pumping
MT-CO1	Cytochrome c oxidase subunit I	P00395	Complex IV	Catalytic core; binds hemes and copper, site of oxygen reduction and proton pumping
MT-CO2	Cytochrome c oxidase subunit II	P00403	Complex IV	Catalytic core; binds copper (CuA), accepts electrons from cytochrome c
MT-CO3	Cytochrome c oxidase subunit III	P00414	Complex IV	Catalytic core; essential for assembly and stability of Complex IV
MT-ATP6	ATP synthase F0	P00846	Complex V	Core F0 subunit;

	subunit 6 (subunit a)			forms part of the proton channel, essential for proton translocation driving ATP synthesis
MT-ATP8	ATP synthase F0 subunit 8 (subunit A6L)	P03928	Complex V	Core F0 subunit; involved in F0 assembly, stability, and linking proton channel to peripheral stalk components

UniProt IDs are for the canonical human sequences. Functions are summarized from various sources including S3, S4, S5-S30, S44, S132.

IV. Orchestrated Assembly: Building the OXPHOS Machinery

A. The Dual Genetic Origin and the Assembly Challenge

The biogenesis of functional OXPHOS complexes is a remarkably complex process, primarily due to their dual genetic origin: subunits are encoded by both nuclear DNA (nDNA) and mtDNA (with the

exception of Complex II, which is entirely nDNA-encoded). This necessitates a highly sophisticated and coordinated assembly pathway to ensure that subunits synthesized in two distinct cellular compartments—the cytosol for nuclear-encoded proteins and the mitochondrial matrix for mtDNA-encoded proteins—are produced in appropriate stoichiometries, correctly targeted, folded, modified, and ultimately integrated into functional holoenzymes within the IMM.

The 13 mtDNA-encoded subunits are characteristically very hydrophobic. They are synthesized on mitochondrial ribosomes (mitoribosomes) that are often found associated with the IMM, facilitating their co-translational insertion directly into the membrane. This immediate membrane integration is crucial for these hydrophobic proteins. In contrast, the far more numerous nuclear-encoded subunits (over 70 for the OXPHOS system) are synthesized on cytosolic ribosomes and must be imported into the mitochondria. This import process involves a series of sophisticated protein translocase machineries, such as the Translocase of the Outer Membrane (TOM complex) and the Translocase of the Inner Membrane (e.g., TIM23 complex for matrix/IMM proteins, TIM22 complex for IMM carrier proteins). The sheer logistical challenge of coordinating these two separate production lines and ensuring the precise assembly of numerous subunits underscores the necessity for intricate regulatory networks and dedicated assembly factors.

B. Role of mtDNA-Encoded Subunits as Assembly Seeds/Scaffolds

There is compelling evidence that certain mtDNA-encoded subunits act as critical nucleation points or scaffolds for the assembly of their respective complexes, or at least specific modules within them. This

hierarchical assembly often begins with the synthesis and membrane insertion of these key mtDNA-encoded components, around which nuclear-encoded subunits and other mtDNA-encoded partners subsequently coalesce.

For **Complex I**, the assembly pathway involves the formation of distinct membrane-associated and matrix arm modules that later combine. Several mtDNA-encoded subunits are pivotal in this process:

- MT-ND1 appears to be crucial for an early assembly intermediate, and its absence, or that of MT-ND2, leads to the accumulation of partially assembled subcomplexes, indicating their foundational role.
- MT-ND4 and MT-ND6 are particularly critical for the proper assembly of the other mtDNA-encoded subunits into the membrane arm of Complex I. Studies have shown that the absence of either MT-ND4 or MT-ND6 can severely disrupt the formation of the entire membrane arm, highlighting their role as essential "linchpin" subunits for this domain. This establishes a clear intra-mitochondrial dependency, where the correct integration of these specific mtDNA-encoded proteins is a prerequisite for the stable association of other mtDNA-encoded subunits within the same complex.

In the case of **Complex IV**, MT-CO1 is widely regarded as the cornerstone or "step-stone" for assembly. Its synthesis and insertion into the IMM initiate the biogenesis of Complex IV, with MT-CO2 and MT-CO3 being incorporated in subsequent, ordered steps. The synthesis and early assembly stages of MT-CO1 are themselves tightly regulated by a cohort of dedicated assembly factors, forming what is known as the MITRAC (Mitochondrial Translation Regulation

Assembly Intermediate of Cytochrome c oxidase) complex (discussed further in Section VI).

This role as assembly seeds means that the mtDNA-encoded proteins are not just passive structural components but active participants in orchestrating their own complex formation, creating an intrinsic connection based on sequential, ordered assembly.

C. Interactions with Nuclear-Encoded Structural Subunits and Assembly Factors

The successful biogenesis of each OXPHOS complex from its nascent mtDNA- and nDNA-encoded subunits into a functional holoenzyme is a highly chaperoned process, guided by a large and diverse array of nuclear-encoded assembly factors. These factors perform various roles, including facilitating subunit folding, mediating the insertion of prosthetic groups (like hemes and iron-sulfur clusters), promoting membrane insertion, stabilizing assembly intermediates, and preventing the aggregation of orphaned subunits. These assembly factors are critical mediators of the mito-nuclear connection, physically and functionally bridging the two protein synthesis systems by interacting with both newly synthesized mtDNA-encoded subunits and imported nuclear-encoded components.

- **Complex I:** This massive complex, with 14 core subunits (7 mtDNA-encoded, 7 nDNA-encoded) responsible for catalysis and about 31 additional nuclear-encoded "supernumerary" subunits involved in assembly, stability, and regulation, exemplifies this intricate interplay. Direct physical interactions between mtDNA-encoded ND subunits and specific nuclear-encoded subunits are numerous and critical for

structural integrity. For example, NDUFA1 interacts with MT-ND1 and MT-ND6; NDUFA10 primarily interacts with MT-ND2, particularly its C-terminal sector involved in proton translocation; and NDUFA11 contacts MT-ND2, MT-ND4, and MT-ND5. These specific contacts underscore the precise architectural requirements for Complex I assembly.

- **Complex III:** While MT-CYB is the only mtDNA-encoded subunit, its assembly with nuclear-encoded partners like cytochrome c1, Rieske iron-sulfur protein, and core proteins UQCRC1 and UQCRC2 is facilitated by assembly factors such as BCS1L (for Rieske protein incorporation) and LYRM7 (UQCRFS1 stabilization).
- **Complex IV:** The assembly of the three mtDNA-encoded catalytic core subunits (MT-CO1, MT-CO2, MT-CO3) with at least 10 nuclear-encoded subunits is a multistep process. Factors like COX20 and COX18 are involved in the maturation and C-terminal translocation of MT-CO2. Copper delivery and insertion into the active sites of MT-CO1 and MT-CO2 are mediated by a cascade of chaperones including SCO1, SCO2, and COA6. SURF1 is essential for the early stages of MT-CO1 subcomplex formation.
- **Complex V:** The assembly of the F0 sector involves the mtDNA-encoded MT-ATP6 and MT-ATP8. The nuclear-encoded protein TMEM70 plays a crucial role in the formation of the c-subunit ring (itself nuclear-encoded) and its subsequent interaction with MT-ATP6 (subunit a) and MT-ATP8 (subunit A6L/8), and also protects the c-ring from proteolysis.

The co-translational insertion of the highly hydrophobic mtDNA-encoded subunits into the IMM likely facilitates their immediate interaction with these membrane-associated assembly factors and nascent nuclear-encoded partners arriving from the

cytosol. This tight spatiotemporal coupling of synthesis and assembly is thought to enhance efficiency, prevent the misfolding or aggregation of reactive hydrophobic proteins, and provide an early checkpoint for quality control, thereby constituting an immediate and critical structural and functional connection.

V. Higher-Order Connections: Respiratory Supercomplexes (SCs)

Beyond their assembly into individual holoenzymes, Complexes I, III, and IV of the mitochondrial respiratory chain (and sometimes Complex V) can further associate to form large, stable, supramolecular structures known as respiratory supercomplexes (SCs) or respirasomes. Complex II (succinate dehydrogenase) is generally not considered a stable stoichiometric component of these canonical SCs. These SCs represent a "meta-level" of connection, physically linking entire functional units that already contain distinct sets of mtDNA-encoded proteins. This implies that the functional state or assembly status of one complex can directly influence its association with, and potentially the function or stability of, other complexes within the SC.

A. Definition and Composition of Respiratory Supercomplexes

In mammals, several distinct SCs have been characterized, with the most prominent forms including:

- **Respirasome ($I_1III_2IV_1$):** This is the most extensively studied SC and consists of one monomer of Complex I, one dimer of Complex III ($CIII_2$), and one monomer of Complex IV. This structure incorporates all 11 core mtDNA-encoded subunits from these three complexes: the seven ND subunits from Complex I (MT-ND1, -ND2, -ND3, -ND4, -ND4L, -ND5, -ND6), two molecules of MT-CYB from the Complex III dimer, and the three

COX subunits from Complex IV (MT-CO1, -CO2, -CO3).

- **SC I_1III_2:** This SC comprises one monomer of Complex I and a Complex III dimer. It contains a total of nine mtDNA-encoded protein molecules (seven from CI and two MT-CYB from $CIII_2$).
- **SC III_2IV_1 (or III_2IV_2):** This SC consists of a Complex III dimer associated with one or sometimes two monomers of Complex IV. It would thus contain five (two MT-CYB + three MT-CO) or eight (two MT-CYB + six MT-CO) mtDNA-encoded protein molecules, respectively.
- **Megacomplexes/Respiratory Strings:** Even larger assemblies, such as those containing two Complex I units (e.g., $I_2III_2IV_{1-2}$), have also been observed and are sometimes referred to as megacomplexes or respiratory strings.

Additionally, Complex V (ATP synthase) is known to form dimers and higher-order oligomers, which are crucial for shaping the mitochondrial cristae and may also influence its own activity and stability.

B. Functional Significance of Supercomplexes

The physiological roles of SCs are subjects of ongoing research, but several functional advantages have been proposed:

- **Substrate Channeling:** SCs are thought to enhance the efficiency of electron transfer by creating micro-compartments or channels for the mobile electron carriers ubiquinone (between CI/CII and CIII) and cytochrome c (between CIII and CIV). This channeling would minimize the diffusion distances of these carriers, prevent their loss to side reactions, and increase the overall kinetic efficiency of the respiratory chain. This directly connects the function of mtDNA-encoded subunits

located in different complexes by optimizing the flow of electrons between them.

- **Stabilization of Individual Complexes:** The formation of SCs may confer structural stability to their constituent complexes, particularly Complex I, which can be labile when isolated. This implies a structural interdependency that benefits all participating complexes and their subunits.
- **Reduced Reactive Oxygen Species (ROS) Production:** By optimizing electron flow and minimizing electron leakage, SCs might contribute to a reduction in the production of deleterious ROS by the respiratory chain.
- **Assembly Intermediates or Platforms:** SCs, or pre-supercomplex assemblies, might serve as platforms for the final maturation or assembly steps of individual complexes, ensuring their correct integration into the respiratory chain.
- **Dynamic Regulation (Plasticity Model):** The relative abundance and composition of different SCs can vary between tissues, developmental stages, and in response to different metabolic or physiological conditions (e.g., hypoxia, nutrient availability). This "plasticity model" suggests that SCs are not static entities but dynamic structures that allow mitochondria to adapt their respiratory machinery to changing cellular demands. This dynamic nature implies that the connections between OXPHOS complexes are themselves regulated.

C. Assembly of Supercomplexes and Role of Specific Factors

The mechanisms governing SC assembly are still being elucidated. Two main models have been proposed:

- **Sequential Assembly Model:** Fully assembled and functional individual OXPHOS complexes diffuse within the IMM and then

associate to form SCs.

- **Co-assembly/Maturation Model:** SCs may begin to form from partially assembled complexes, or the formation of an SC might be a prerequisite for the final maturation and activation of one or more of its constituent complexes. For instance, there is evidence suggesting that the maturation of Complex I might depend on its association with a Complex III dimer and Complex IV in a "pre-respirasome" scaffold.

Several factors are known to influence SC formation and stability:

- **Cardiolipin:** This mitochondria-specific phospholipid, with its unique conical shape and four acyl chains, is thought to play a crucial role in stabilizing individual respiratory complexes and promoting their association into SCs by acting as a form of "molecular glue" at complex interfaces.
- **SCAFI (Supercomplex Assembly Factor I, also known as COX7A2L):** This nuclear-encoded protein is a key factor in SC biogenesis. SCAFI is an isoform of the Complex IV subunit COX7A. Its primary role is to specifically mediate the formation of the SC III_2IV_1 (and SCs containing this module, such as the S-respirasome) by acting as a molecular bridge or tether that physically links Complex III and Complex IV. In doing so, SCAFI directly facilitates a connection between the mtDNA-encoded MT-CYB (in CIII) and the mtDNA-encoded MT-CO1, MT-CO2, and MT-CO3 (in CIV). The differential expression or incorporation of SCAFI versus other COX7A isoforms (like COX7A1 in muscle or the ubiquitously expressed COX7A2) can determine the types and abundance of SCs formed, thereby influencing OXPHOS efficiency and metabolic adaptation strategies of the cell. This makes SCAFI a critical, genetically encoded mediator of inter-complex connectivity.

- **COA1 (Cytochrome c Oxidase Assembly Factor 1, also known as MITRAC15):** While primarily recognized as a Complex IV assembly factor involved in the early stages of MT-CO1 biogenesis (see MITRAC complex, Section VI), COA1 has also been found to be associated with Complex I assembly intermediates. This dual association suggests a potential role for COA1 in coordinating the biogenesis of Complex I and Complex IV, possibly facilitating their co-assembly into larger SC structures or ensuring balanced production for SC formation.

D. Table 2: mtDNA-Encoded Subunits in Major Human Respiratory Supercomplexes

Supercomplex Stoichiometry	**mtDNA-Encoded Subunits from Complex I**	**mtDNA-Encoded Subunit from Complex III (MT-CYB)**	**mtDNA-Encoded Subunits from Complex IV (MT-CO1, -CO2, -CO3)**	**Total mtDNA-Encoded Protein Molecules per SC**
Respirasome ($I_1III_2IV_1$)	MT-ND1, ND2, ND3, ND4, ND4L, ND5, ND6	2 (from CIII dimer)	MT-CO1, CO2, CO3	12
SC I_1III_2	MT-ND1, ND2, ND3, ND4, ND4L, ND5, ND6	2 (from CIII dimer)	N/A	9
SC III_2IV_1	N/A	2 (from CIII	MT-CO1,	5

		dimer)	CO2, CO3	
SC III_2IV_2	N/A	2 (from CIII dimer)	2 x (MT-CO1, CO2, CO3)	8
Megacomplex (e.g., $I_2III_2IV_1$)	2 x (MT-ND1, ND2, ND3, ND4, ND4L, ND5, ND6)	2 (from CIII dimer)	MT-CO1, CO2, CO3	19

Based on information from S51, S89, S90, B10, S131, S170. 'N/A' indicates the complex is not part of that specific SC.

VI. Coordinated Regulation: Ensuring Balanced Expression of mtDNA-Encoded Proteins

The synthesis of the 13 mtDNA-encoded proteins must be precisely coordinated with each other and with the synthesis and import of over 70 nuclear-encoded OXPHOS subunits to ensure the stoichiometric assembly of functional respiratory complexes. This coordination is largely achieved through intricate post-transcriptional regulatory mechanisms, as the mitochondrial genome's unique transcriptional strategy offers limited gene-specific control at the initiation level.

A. Polycistronic Transcription and mt-RNA Processing

Human mtDNA is transcribed from a small number of promoters located in the main non-coding region (NCR), also known as the D-loop. The heavy strand promoter (HSP) and light strand promoter (LSP) drive the synthesis of long, polycistronic precursor RNAs that encompass nearly the entire respective strands. These precursors contain the sequences for mRNAs, rRNAs, and tRNAs.

The individual RNA species are liberated from these polycistronic transcripts primarily through the "tRNA punctuation model". According to this model, the tRNA sequences interspersed among the mRNA and rRNA sequences are recognized and precisely excised by endonucleolytic enzymes: RNase P cleaves at the 5' end of the tRNAs, and ELAC2 (also known as RNase Z) cleaves at their 3' end. This process releases mature tRNAs and, concomitantly, the individual mRNAs and rRNAs. This shared pathway of co-transcription and co-processing via tRNA excision forms a fundamental connection point in the biogenesis of all mtDNA-encoded gene products.

Following excision, mt-mRNAs undergo further maturation, including the addition of a short poly(A) tail by the mitochondrial poly(A) polymerase (mtPAP). Conversely, non-coding RNAs like tRNAs and rRNAs that may acquire spurious poly(A) extensions during processing have these tails removed by the phosphodiesterase PDE12, which is crucial for their proper function.

B. Post-Transcriptional Regulons and Coordinated Control

Despite being transcribed as part of large polycistrons, the steady-state levels of individual mt-mRNAs and, consequently, their protein products, can vary significantly. This variation underscores the critical importance of post-transcriptional regulatory mechanisms in fine-tuning the expression of each mtDNA-encoded protein to meet the stoichiometric demands of OXPHOS complex assembly. This multi-layered post-transcriptional control serves as the primary hub for establishing differential expression and thus functional connections related to balanced subunit production.

1. Mitochondrial RNA Granules (MRGs): Hubs for mt-RNA Metabolism
Mitochondrial RNA Granules (MRGs) are dynamic, membraneless foci within the mitochondrial

matrix that serve as critical centers for mt-RNA metabolism. These granules concentrate newly transcribed mtRNAs along with a plethora of nuclear-encoded proteins involved in various aspects of RNA maturation, including processing, modification, quality control, degradation, and the assembly of mitoribosomes. Key protein components of MRGs include RNA-binding proteins like GRSF1, members of the FASTKD family (e.g., FASTKD1, which regulates MT-ND3 mRNA levels), the mitochondrial poly(A)-polymerase (mtPAP), various RNA helicases (e.g., DHX30), and components of the mitochondrial degradosome (SUV3-PNPase) involved in RNA turnover.

MRGs are thought to provide spatiotemporal organization to the complex cascade of mt-RNA processing events. By co-localizing multiple mt-RNA species with the necessary enzymatic machinery, MRGs can facilitate the coordinated processing and maturation of transcripts destined to become the 13 mRNAs, 22 tRNAs, and 2 rRNAs. This co-localization within MRGs establishes a physical and functional connection hub for the biogenesis of all mtDNA-encoded transcripts, as defects in MRG components or function can broadly impact mitochondrial gene expression. Recent research also suggests the formation of distinct "inhibition granules" under conditions of transcription arrest, which may serve to protect and stabilize certain mt-mRNAs.

2. Nuclear-Encoded RNA-Binding Proteins (RBPs) and Translational Activators
A host of nuclear-encoded proteins are imported into mitochondria to directly interact with mt-RNAs and modulate their fate.

- **LRPPRC (Leucine-Rich Pentatricopeptide Repeat Containing Protein):** LRPPRC is a crucial post-transcriptional regulator that forms a complex with SLIRP (SDBP, Stem-loop RNA Binding Protein) within mitochondria. This complex is essential for the stability and proper polyadenylation of virtually all mitochondrial mRNAs. Depletion or mutation of LRPPRC leads to a dramatic reduction in the steady-state levels of most, if not all, mt-mRNAs, profoundly impairing mitochondrial translation. Pathogenic mutations in *LRPPRC* cause Leigh Syndrome French-Canadian type (LSFC), a devastating neurological

disorder characterized by a severe, combined OXPHOS deficiency due to the reduced expression of multiple mtDNA-encoded subunits, particularly affecting Complexes IV and I. LRPPRC thus acts as a global regulator, connecting the stability and processing of the cohort of 13 protein-coding mt-mRNAs.

- **TACO1 (Translational Activator of Cytochrome C Oxidase I):** Initially identified as a specific translational activator for *MT-CO1* mRNA, recent studies suggest a more nuanced role for TACO1. It appears to be required for the efficient translation of polyproline-rich sequences found in both MT-CO1 and MT-CO3, alleviating mitoribosome stalling at these challenging motifs. Its impact on the translation of other mt-mRNAs seems to be negligible. Mutations in *TACO1* cause an isolated Complex IV deficiency and are associated with specific forms of Leigh Syndrome. TACO1 therefore provides a specific connection for the efficient co-production of two critical mtDNA-encoded subunits of Complex IV.
- **Other Nuclear Factors:** Numerous other nuclear-encoded factors are indispensable for the expression of all 13 mtDNA-encoded proteins, thereby creating a connection through shared essential machinery. These include the mitochondrial poly(A) polymerase (MTPAP), the deadenylase PDE12, the full complement of mitoribosomal proteins, and various mitochondrial translation initiation, elongation, and termination factors. Furthermore, genome-wide association studies have identified nuclear loci, such as those containing *TBRG4*, *MTPAP*, and *LONP1*, whose variants are associated with the expression levels of multiple mitochondrial genes, hinting at further layers of coordinated *trans*-acting regulation.

3. The MITRAC System (Mitochondrial Translation Regulation Assembly Complex)
The MITRAC system represents a sophisticated quality control mechanism that tightly couples the translation of specific mtDNA-encoded subunits with their assembly into mature OXPHOS complexes, particularly for Complex IV and its cornerstone subunit, MT-CO1. MITRAC is not a single static complex but rather a series of dynamic assembly intermediates. Key components include the mtDNA-encoded MT-CO1 itself, and nuclear-encoded factors such as MITRAC12 (COA3), C12orf62 (COX14), MITRAC15 (COA1), TIM21 (a subunit of the TIM23 import machinery), and MITRAC7.

MITRAC components, notably C12orf62 and MITRAC12, directly interact with mitoribosomes actively synthesizing MT-CO1. This interaction allows the MITRAC system to sense the availability of incoming nuclear-encoded Complex IV subunits (e.g., COX4) that are imported via the TIM23 machinery. If nuclear-encoded partners are scarce, MT-CO1 translation can be attenuated or paused, preventing the accumulation of orphaned, potentially harmful, mtDNA-encoded subunits. Once the nuclear partners become available and are incorporated, translation of MT-CO1 can resume to completion, and the nascent MT-CO1 is channeled into subsequent assembly steps. This "just-in-time" production system provides a direct physical and regulatory link between the synthesis of mtDNA-encoded COX subunits and their assembly with nuclear-encoded partners, ensuring stoichiometric balance and efficient biogenesis of Complex IV.

C. Mitochondrial-Nuclear Crosstalk and Co-evolution

The successful construction and operation of the OXPHOS system, with its dual genetic origin, mandates continuous and robust communication between the mitochondrial and nuclear compartments. This mito-nuclear crosstalk ensures that the expression of genes from both genomes is coordinated to meet cellular energy demands and respond to various stresses.

- **Retrograde Signaling:** Mitochondria are not passive recipients

of nuclear control; they can actively signal their functional status (e.g., OXPHOS efficiency, levels of ATP or ROS, integrity of mtDNA) to the nucleus. This "retrograde signaling" can modulate nuclear gene expression programs, for instance, by upregulating nuclear genes encoding OXPHOS subunits, assembly factors, or antioxidant enzymes in response to mitochondrial dysfunction. Novel mtDNA-encoded peptides like Humanin have been implicated as potential mediators of such retrograde signals, providing a direct feedback loop from the mitochondrial genome to the nucleus.

- **Co-evolutionary Pressures:** Mitochondrial DNA typically exhibits a higher mutation rate compared to nuclear DNA. This disparity imposes a constant evolutionary pressure on nuclear genes that encode proteins interacting with mtDNA-encoded products (i.e., other OXPHOS subunits, assembly factors, translational machinery components). These nuclear genes must co-evolve to maintain functional compatibility with their rapidly evolving mitochondrial partners, ensuring the continued integrity of essential interactions. This co-evolutionary dynamic is a fundamental, long-term connection that shapes the entire OXPHOS system.
- Key transcriptional co-activators like PGC-1α (Peroxisome proliferator-activated receptor gamma coactivator 1-alpha) and transcription factors like CEBPB (CCAAT/enhancer-binding protein beta) are known master regulators of mitochondrial biogenesis and are implicated in coordinating the expression of both mitochondrial and nuclear genes involved in OXPHOS in response to various physiological cues, such as exercise or developmental transitions.

This intricate network of regulatory mechanisms, spanning from

polycistronic RNA processing to specific translational activation and intergenomic communication, ensures that the 13 mtDNA-encoded proteins are produced in a balanced and responsive manner, ultimately connecting their fates to the overall metabolic state of the cell.

D. Table 3: Key Nuclear-Encoded Factors Co-regulating Multiple mtDNA-Encoded Proteins

Factor Name	Type of Factor	Primary Target mt-RNAs/Proteins or Process	Key Regulatory Role	Associated Disease (if applicable)
LRPPRC	RNA-binding protein	Most/all mt-mRNAs	mRNA stability, polyadenylation, coordination of mitochondrial translation	Leigh Syndrome French-Canadian (LSFC)
SLIRP (SDBP)	RNA-binding protein	Most/all mt-mRNAs (in complex with LRPPRC)	mRNA stability, protection from degradation	(Associated with LRPPRC dysfunction)
TACO1	Translational activator	MT-CO1, MT-CO3 mRNAs	Translation elongation efficiency,	Leigh Syndrome (Complex IV

		(polyproline-rich regions)	prevention of mitoribosome stalling	deficiency)
PDE12	Phosphodiesterase	mt-tRNAs, mt-rRNAs (aberrant poly(A) tails)	RNA processing, removal of spurious poly(A) tails, ensuring tRNA/rRNA functionality	Mitochondrial disease (e.g., encephalopathy)
mtPAP	Mitochondrial Poly(A) Polymerase	mt-mRNAs	mRNA maturation, polyadenylation	Spastic ataxia, optic atrophy
GRSF1	RNA-binding protein (MRG component)	Nascent mt-RNAs, specific mt-mRNAs	RNA processing, stabilization, localization to MRGs	(Implicated in mt-RNA metabolism)
FASTKD1	RNA-binding protein (MRG component)	MT-ND3 mRNA	Regulation of MT-ND3 mRNA levels, mt-RNA processing in MRGs	(Component of MRGs)
C12orf62 (COX14)	MITRAC component, Assembly	MT-CO1 translation and	Coupling MT-CO1 synthesis to	Leigh Syndrome (Complex IV

	factor	assembly	Complex IV assembly, interaction with nascent MT-CO1 on mitoribosomes	deficiency)
MITRAC12 (COA3)	MITRAC component, Assembly factor	MT-CO1 translation and assembly	Early steps in MT-CO1 biogenesis, interaction with nascent MT-CO1	Complex IV deficiency
TIM21	MITRAC component, TIM23 complex subunit	Assembly of MT-CO1, MT-CO2, MT-CO3 (CIV); MT-ND2, ND4, ND5 (CI); MT-CYB (CIII)	Links protein import with OXPHOS complex assembly, coordinates nuclear and mitochondrial subunit integration	(Potential for broad OXPHOS defects)
PGC-1α	Transcriptional co-activator	Nuclear genes for OXPHOS, mtDNA replication/transcription factors	Master regulator of mitochondrial biogenesis, coordinated mito-nuclear gene	(Dysregulation in metabolic diseases)

			expression	
SUV3 / PNPase	RNA Helicase / Polynucleotide Phosphorylase (Degradosome components)	All mt-RNAs	mt-RNA surveillance and degradation	(Mutations can lead to mt-RNA accumulation)

This table summarizes information from sources including S1, S10, S49, S70, S73, S121, S123-S126, S130, S133-S137, S139-S148, S163-S166, S171, S181-S184, B7, B13, B17, B18, B23.

VII. Broader Interaction Networks and Cellular Roles

The 13 mtDNA-encoded proteins, primarily through their incorporation into the five OXPHOS complexes, are embedded within a much larger network of molecular interactions that extend beyond the immediate confines of energy production. These broader connections link mitochondrial bioenergetics to diverse cellular processes, including inter-organellar communication, signaling pathways, and responses to cellular stress.

A. Insights from Large-Scale Proteomic and Genetic Interaction Studies

The advent of high-throughput technologies has enabled the systematic mapping of protein-protein interactions (PPIs) and genetic interactions on a genome-wide scale. Databases such as STRING (Search Tool for the Retrieval of Interacting Genes/Proteins),

BioGRID (Biological General Repository for Interaction Datasets), and IntAct curate and consolidate these interaction data, providing valuable resources for exploring the connectivity of mitochondrial proteins. The MitoCarta inventory, for instance, has cataloged over a thousand human genes encoding proteins with strong evidence of mitochondrial localization, highlighting the vast network of nuclear-encoded proteins that support, regulate, or interact with the mitochondrial proteome, including the 13 mtDNA-encoded components.

Proximity-dependent biotinylation assays, such as BioID, have emerged as powerful tools for identifying transient or weak interactions and mapping protein interaction networks within specific cellular compartments or sub-organellar regions. A notable study by Antonicka et al. (2020) utilized BioID with 100 distinct mitochondrial bait proteins, generating a high-density human mitochondrial proximity interaction network comprising over 15,000 high-confidence interactions among nearly 1,500 proteins. This extensive dataset, covering approximately half of the known mitochondrial proteome as defined by MitoCarta2.0, provides unprecedented insights into the organization and connectivity of mitochondrial protein modules. While directly using the 13 mtDNA-encoded proteins as baits in such assays is technically challenging due to their mitochondrial synthesis and hydrophobic nature, interactions involving their assembled OXPHOS complexes can be inferred when nuclear-encoded subunits or closely associated assembly factors are used as baits. This approach allows for the elucidation of a broader interaction landscape for the 13 proteins via their stable incorporation into the OXPHOS machinery, potentially revealing links to proteins involved in metabolite transport, mitochondrial quality control, organelle dynamics, and

signaling events at inter-organellar contact sites.

B. OXPHOS Complexes/Subunits at Mitochondria-ER Contact Sites (MAMs)

Mitochondria-associated ER membranes (MAMs) are specialized microdomains where the endoplasmic reticulum (ER) and the outer mitochondrial membrane (OMM) come into close apposition (typically 10-30 nm apart). These contact sites are not sites of membrane fusion but are bridged by specific tethering proteins, creating a unique platform for direct inter-organellar communication and the exchange of molecules such as calcium ions (Ca^{2+}) and lipids. MAMs play crucial roles in a wide array of cellular processes, including Ca^{2+} homeostasis, lipid synthesis and trafficking, regulation of mitochondrial dynamics (fission and fusion), apoptosis, ER stress responses, and autophagy/mitophagy.

The functional integrity of OXPHOS complexes, and thus the activity of their 13 mtDNA-encoded subunits, is closely intertwined with MAM function:

- **Calcium Signaling:** One of the best-characterized functions of MAMs is the efficient transfer of Ca^{2+} from ER stores to the mitochondrial matrix. Mitochondrial Ca^{2+} uptake stimulates the activity of several dehydrogenases in the tricarboxylic acid (TCA) cycle, increasing the supply of NADH and $FADH_2$ to the electron transport chain. Furthermore, elevated matrix Ca^{2+} can directly or indirectly modulate the activity of OXPHOS complexes themselves, thereby enhancing ATP production. This creates a direct functional link where the performance of the OXPHOS system, reliant on the 13 mtDNA-encoded proteins, is responsive to Ca^{2+} signals relayed through MAMs.

- **Proteomic Evidence:** Proteomic analyses of isolated MAM fractions have revealed an enrichment of proteins associated with mitochondrial function and energy metabolism. While these studies may not always pinpoint specific OXPHOS subunits as integral structural components of the MAM tethering machinery itself, the functional data strongly suggest that OXPHOS complexes are either strategically localized in close proximity to MAMs or their activity is tightly coupled to processes occurring at these interfaces. This proximity would facilitate rapid responses to changes in Ca^{2+} flux or lipid transfer.
- **MAM Disruption and OXPHOS Dysfunction:** Experimental disruption of MAM integrity or alterations in the expression of key MAM-associated proteins (e.g., Mitofusin 2 (MFN2), a protein involved in both mitochondrial fusion and ER-mitochondria tethering) can lead to mitochondrial dysfunction, including impaired Ca^{2+} handling, altered mitochondrial morphology, and defects in OXPHOS, such as reduced Complex I activity or aberrant electron flow.

The close functional relationship between MAMs and OXPHOS suggests that the 13 mtDNA-encoded proteins, as core components of the energy-transducing machinery, are key players in the dialogue between mitochondria and the ER, allowing cellular energy status to be integrated with ER-centric signaling pathways and lipid metabolism.

C. Other Potential Interactions and Roles

Beyond their canonical roles within OXPHOS complexes and their functional links to MAMs, individual mtDNA-encoded subunits may engage in more specific interactions that connect them to other

cellular pathways or disease processes.

- **MT-CO3 Interactions:** The MT-CO3 subunit of Complex IV has been reported to potentially interact with several interesting proteins:
    - **SNCA (α-synuclein):** α-synuclein is a protein centrally implicated in the pathogenesis of Parkinson's disease. It is known to accumulate in Lewy bodies and can localize to mitochondria, where it may impair mitochondrial function, dynamics, and quality control. A direct interaction between MT-CO3 and α-synuclein could provide a molecular link between Complex IV dysfunction and the neurodegenerative processes in Parkinson's disease, perhaps by α-synuclein directly modulating Complex IV activity or stability, or by MT-CO3 influencing α-synuclein aggregation or localization.
    - **KRAS and RAC1:** These are small GTPases that act as critical molecular switches in signaling pathways regulating cell growth, proliferation, differentiation, and cytoskeletal dynamics. While the functional context of a direct interaction with an OXPHOS subunit like MT-CO3 is not immediately obvious from the provided materials, it could speculatively imply a mechanism for sensing or responding to cellular energy status by these core signaling pathways, or conversely, a way for these pathways to modulate mitochondrial respiration.
    - **HSPB2 (Heat Shock Protein Family B Member 2):** HSPB2 is a small heat shock protein predominantly expressed in cardiac and skeletal muscle. It has been shown to interact with the outer mitochondrial membrane and is thought to play a role in protecting mitochondrial energetics, particularly under stress conditions like ischemia. An

interaction with MT-CO3, an inner membrane protein, might suggest a role for HSPB2 in the assembly, stability, or stress-induced quality control of Complex IV.

These potential interactions, particularly if validated and functionally characterized within the context of OXPHOS and cellular physiology, would represent novel connections for MT-CO3, expanding its functional repertoire beyond its established role within Complex IV. Such specific interactions between an individual mtDNA-encoded protein and key players in distinct disease pathways (like SNCA in Parkinson's) could offer valuable insights into how mitochondrial dysfunction contributes to broader cellular pathology beyond simple ATP depletion.

VIII. Pathophysiological Ramifications: When Connections Are Compromised

The intricate web of functional, structural, and regulatory connections among the 13 mtDNA-encoded proteins and their nuclear-encoded partners means that disruptions at any level can have profound pathological consequences. These disruptions manifest primarily as mitochondrial diseases, a heterogeneous group of disorders often characterized by impaired energy production and multi-systemic involvement, particularly affecting tissues with high energy demands such as the brain, skeletal muscle, heart, liver, and sensory organs. The wide range of clinical phenotypes observed underscores the systemic importance and interconnectedness of the OXPHOS system; a defect in one component can have ripple effects, impacting cellular function in diverse ways.

A. Diseases Linked to Mutations in mtDNA-Encoded Proteins

Direct mutations within the 13 protein-coding genes of mtDNA are

well-established causes of numerous human mitochondrial diseases. These mutations can affect the synthesis, stability, assembly, or catalytic function of the encoded subunit, leading to a deficiency in the respective OXPHOS complex and, consequently, the entire respiratory chain. Examples include:

- **Leber Hereditary Optic Neuropathy (LHON):** Primarily characterized by acute or subacute bilateral loss of central vision, LHON is most commonly associated with point mutations in *MT-ND1*, *MT-ND4*, *MT-ND4L*, *MT-ND5*, or *MT-ND6* (all encoding Complex I subunits). Less frequently, mutations in *MT-CYB* (Complex III) or *MT-CO3* (Complex IV) have been implicated.
- **Mitochondrial Encephalomyopathy, Lactic Acidosis, and Stroke-like Episodes (MELAS):** While often caused by mutations in mt-tRNA genes (most commonly *MT-TL1*, encoding tRNALeu(UUR)), pathogenic point mutations in protein-coding genes such as *MT-ND1*, *MT-ND5*, *MT-ND6* (Complex I), and *MT-CO3* (Complex IV) can also give rise to MELAS or MELAS-like phenotypes.
- **Leigh Syndrome (Subacute Necrotizing Encephalomyelopathy):** This severe, progressive neurodegenerative disorder of infancy or early childhood can result from mutations in a wide range of mtDNA-encoded genes, including *MT-ND3*, *MT-ND5*, *MT-ND6* (Complex I), *MT-CO3* (Complex IV), and notably *MT-ATP6* (Complex V).
- **Cardiomyopathies and Myopathies:** Mutations in *MT-CYB* can lead to isolated myopathy or hypertrophic cardiomyopathy (HCM). Defects in *MT-ATP6* and *MT-ATP8* are linked to Neuropathy, Ataxia, and Retinitis Pigmentosa (NARP), Maternally Inherited Leigh Syndrome (MILS), and various cardiomyopathies.

Mutations in *MT-CO1* have been associated with sensorineural deafness, and *MT-CO3* mutations with recurrent myoglobinuria.

These examples demonstrate that the functional integrity of each of the 13 mtDNA-encoded proteins is critical, and their individual failures can cripple the entire OXPHOS pathway, highlighting their profound functional interconnectedness.

B. Impact of Disrupted Co-regulation and Assembly

Given the reliance on a vast array of nuclear-encoded factors for the expression, processing, translation, and assembly of mtDNA-encoded proteins, mutations in these *trans*-acting nuclear genes frequently lead to mitochondrial dysfunction. Such defects often result in combined OXPHOS deficiencies because a single faulty nuclear factor can impact the biogenesis or stability of multiple mtDNA-encoded subunits or even entire complexes. This clearly illustrates a hierarchical connection where a single point of failure in a shared regulatory or assembly pathway can unravel the coordinated production of many of the 13 proteins.

- **LRPPRC Deficiency (Leigh Syndrome French-Canadian type - LSFC):** Mutations in the *LRPPRC* gene, which encodes a protein crucial for the stability and polyadenylation of most, if not all, mitochondrial mRNAs, lead to LSFC. The resulting global instability of mt-mRNAs causes a severe combined deficiency of OXPHOS complexes, with Complex IV (cytochrome c oxidase) and Complex I often being the most affected. This pathology starkly demonstrates how a defect in a single factor that connects the expression of all 13 proteins can have devastating systemic consequences.
- **TACO1 Deficiency:** Mutations in *TACO1*, which encodes a

translational activator for MT-CO1 and MT-CO3, primarily result in an isolated Complex IV deficiency and are associated with specific forms of Leigh Syndrome. Although more targeted than LRPPRC defects, TACO1 dysfunction still disrupts the coordinated synthesis of two key mtDNA-encoded subunits within the same complex.

- **MITRAC Complex Defects:** The MITRAC system is essential for coupling MT-CO1 translation with Complex IV assembly. Mutations in genes encoding MITRAC components, such as *SURF1*, *COA1* (MITRAC15), *C12orf62* (COX14), or *MITRAC12* (COA3), typically cause isolated Complex IV deficiency and can lead to Leigh Syndrome or other severe neurological presentations. Some MITRAC-associated proteins, like MITRAC15 (COA1) or TIM21, may have broader roles, potentially influencing Complex I assembly as well, suggesting that defects in these components could lead to more complex OXPHOS deficiencies.
- **Mitochondrial RNA Granule (MRG) Dysfunction:** MRGs are hubs for mt-RNA processing and mitoribosome biogenesis. Impairment of MRG function, whether due to mutations in constituent proteins (e.g., GRSF1, FASTKD family members) or exposure to mitochondrial toxicants like manganese, can disrupt the maturation and stability of mt-transcripts, leading to defective OXPHOS complex assembly and function. This highlights the importance of these shared processing centers for the coordinated expression of the 13 proteins.
- **Supercomplex Assembly Factor Defects:** While the direct disease implications of mutations in dedicated supercomplex assembly factors like SCAFI/COX7A2L are still being fully elucidated in humans, defects in factors that affect the stability or assembly of individual complexes (which then participate in

SCs) can indirectly lead to altered SC profiles. Given the proposed roles of SCs in enhancing OXPHOS efficiency and stability, their destabilization or improper formation due to defects in constituent complexes or specific assembly factors can contribute to the pathomechanism of mitochondrial diseases.

The pathologies arising from disruptions in these connecting machineries underscore that the 13 mtDNA-encoded proteins do not function in isolation but are part of a highly integrated system. Their connections, forged through shared regulatory pathways and assembly processes, are critical for cellular health.

IX. Concluding Remarks: The Intricate Web of Mitochondrial Protein Connectivity

The 13 canonical proteins encoded by the human mitochondrial genome, along with potentially other recently identified peptides, form an intricate and deeply interconnected network that is fundamental to cellular life. Their connections are not merely incidental but are woven into the fabric of their biogenesis, structure, function, and regulation, extending from the molecular intricacies of gene expression to the supramolecular organization of the oxidative phosphorylation system and its integration with broader cellular physiology.

A. Recap of Multifaceted Connections

The exploration of the relationships among these mtDNA-encoded proteins reveals a multifaceted web of connectivity:

- **Shared Functional Destiny in OXPHOS:** All 13 canonical proteins are indispensable core subunits of the OXPHOS complexes, participating sequentially in the electron transport

chain and ATP synthesis. This shared role in cellular energy production is their most fundamental connection.

- **Structural Integration:** They are physically co-assembled into four of the five OXPHOS enzyme complexes. Furthermore, these individual complexes (I, III, and IV) associate to form respiratory supercomplexes, bringing together distinct sets of mtDNA-encoded proteins into even larger functional units.
- **Coordinated Biogenesis via Polycistronic Transcription and Shared Processing:** The transcription of mtDNA as polycistronic units and the subsequent processing of these precursors, often via the tRNA punctuation model, inherently links the biogenesis of all mt-mRNAs, mt-tRNAs, and mt-rRNAs. Shared maturation enzymes like mtPAP and PDE12 further underscore this linkage.
- **Co-regulation by Nuclear-Encoded *Trans*-Acting Factors:** A vast array of nuclear-encoded proteins, including RNA-binding proteins (e.g., LRPPRC), specific translational activators (e.g., TACO1), components of mitochondrial RNA granules (e.g., GRSF1, FASTKD proteins), and factors coupling translation to assembly (e.g., the MITRAC system), govern the post-transcriptional fate of mt-mRNAs. These factors often act on multiple mt-transcripts or coordinate key steps in their expression, thereby connecting the production of the 13 proteins.
- **Hierarchical Interdependence in Assembly:** The assembly of OXPHOS complexes is often a hierarchical process, where the correct synthesis and insertion of certain mtDNA-encoded "seed" subunits (e.g., MT-ND1, MT-ND6 for Complex I; MT-CO1 for Complex IV) are prerequisites for the stable incorporation of other mtDNA-encoded and nuclear-encoded partners.

- **Broader Network Interactions:** Beyond their roles in OXPHOS, these proteins, as part of their complexes, are increasingly recognized to participate in wider cellular networks. This includes functional links to mitochondria-ER contact sites (MAMs), where OXPHOS activity can be modulated by Ca^{2+} signaling and lipid exchange, and potential direct interactions of specific subunits (e.g., MT-CO3 with α-synuclein) with proteins involved in other cellular pathways and disease states.
- **Co-evolution with the Nuclear Genome:** The dual genetic origin of the OXPHOS system necessitates a continuous co-evolutionary dialogue between the mitochondrial and nuclear genomes to maintain the functional integrity of interacting subunits and regulatory systems.
- **Shared Vulnerability and Pathophysiological Manifestations:** Defects in any of the 13 proteins, or in the machinery responsible for their expression and assembly, often lead to a spectrum of mitochondrial diseases with overlapping clinical features, reflecting the systemic impact of disrupting this interconnected energy-producing network.

B. Future Directions for Research

Despite significant advances, our understanding of the full extent and nuances of the connections among mtDNA-encoded proteins is far from complete. Several key areas warrant further investigation:

- **Novel mtDNA-Encoded Peptides:** Continued research is needed to definitively identify and functionally characterize any additional peptides encoded by human mtDNA (such as Humanin and GAU) and to elucidate their roles and potential interactions with the canonical OXPHOS proteins or their regulatory pathways.

- **Tissue-Specific Regulation and Supercomplex Dynamics:** The mechanisms underlying tissue-specific differences in the expression of mtDNA-encoded genes, the composition of OXPHOS complexes, and the dynamic regulation of respiratory supercomplexes in response to varying physiological states (e.g., nutrient availability, hypoxia, exercise) require deeper exploration. Understanding how factors like LRPPRC, TACO1, and SCAFI are themselves regulated in a tissue-specific manner will be crucial.
- **Comprehensive Interactome Mapping:** While challenging, efforts to map the direct and indirect interaction network of all 13 mtDNA-encoded proteins, potentially using advanced proteomic techniques adapted for mitochondrial-synthesized proteins or by systematically baiting their closest nuclear-encoded partners, would provide a more complete picture of their connectivity. The rich dataset from studies like Antonicka et al. (2020) provides a foundation that can be further mined and expanded.
- **OXPHOS at Inter-Organellar Contact Sites:** The precise structural and functional roles of OXPHOS complexes and their individual subunits at mitochondria-ER contact sites (MAMs) and potentially other inter-organellar interfaces need to be more clearly defined. Understanding how these connections integrate mitochondrial energy status with global cellular signaling is a key frontier.
- **Mechanisms of Coordinated Mito-Nuclear Gene Expression:** Unraveling the full complement of nuclear factors (e.g., RNA-binding proteins, translational regulators, signaling molecules involved in retrograde response) that ensure the coordinated expression of mitochondrial and nuclear genes for

OXPHOS biogenesis remains a critical goal. The role of mitochondrial RNA granules in orchestrating these processes warrants further detailed study.

- **Therapeutic Strategies:** A more profound understanding of the intricate connections within the mitochondrial proteome, particularly how they are disrupted in disease, could pave the way for novel therapeutic strategies. Targeting shared regulatory nodes, specific assembly pathways, or factors involved in supercomplex stability might offer new avenues to ameliorate the consequences of mitochondrial dysfunction.

In conclusion, the 13 proteins encoded by the human mitochondrial genome are not isolated entities but are deeply interwoven through a complex hierarchy of functional, structural, and regulatory relationships. Their collective performance is essential for cellular vitality, and the ongoing elucidation of their intricate connections continues to reveal fundamental principles of mitochondrial biology and human health.